

Transmission of *Grapevine Leafroll-associated Virus 3* (GLRaV-3): Acquisition, Inoculation and Retention by the Mealybugs *Planococcus ficus* and *Pseudococcus longispinus* (Hemiptera: Pseudococcidae)

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The vine mealybug, *Planococcus ficus* (Signoret), and the longtailed mealybug, *Pseudococcus longispinus* (Targioni Tozzetti), are vectors of grapevine leafroll-associated virus 3 (GLRaV-3), one of the most abundant viruses associated with grapevine leafroll disease. To elucidate the transmission biology in South Africa, acquisition access periods (AAPs), inoculation access periods (IAPs) and the retention of the virus in starving and feeding first- to second instar nymphs were determined. The rootstock hybrid LN33 served as virus source and grapevines (*Vitis vinifera* L., cv. Cabernet franc) served as recipient plants. An AAP of 15 min or an IAP of 15 min was sufficient for *Pl. ficus* to acquire or transmit GLRaV-3, respectively. Nymphs of *Pl. ficus* retained the virus for at least eight days when feeding on a non-virus host and grapevine, and for at least two days when starving, and were then capable of transmitting it successfully to healthy grapevine plants. Nymphs of *Ps. longispinus* transmitted the virus after an AAP of 30 min and an IAP of 1 h. They retained the virus for at least three days when feeding on virus-free vines or starving. The GLRaV-3 infection rates of plants with *Pl. ficus* as vector varied with AAPs. These were lower (20 to 60%) for AAPs of 12 h or less than for AAPs of 24 h or more (80 to 100%). The findings are of importance for understanding the transmission biology of mealybug vectors and devising management strategies for grapevine leafroll.

INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is one of the most widespread of at least nine viruses associated with grapevine leafroll disease (GLD) (Martin *et al.*, 2005; Pietersen, 2006; Akbaş *et al.*, 2007; Almeida *et al.*, 2013; Naidu *et al.*, 2014). The disease, which affects the quantity and quality of yield (Goheen & Cook, 1959; Cabaleiro *et al.*, 1999; Atallah *et al.*, 2012), has been reported from all major grapevine-growing areas worldwide (Martelli, 1986).

The infection of leafroll-free plant material after planting in open vineyards is a major problem for the grapevine industry (Pietersen *et al.*, 2013). Until the 1980s, GLD was thought to be transmitted by using virus-infected plant material, either as rootstock or in grafts, but then Tanne *et al.* (1989) and Engelbrecht and Kasdorf (1990) showed that the spread of GLRaV-3 in vineyards is also mediated

by mealybugs (Pseudococcidae). Furthermore, a few soft scale (Coccidae) species have been identified as vectors of GLRaV-3 (Belli *et al.*, 1994; Mahfoudhi *et al.*, 2009; Krüger & Douglas-Smit, 2013). Transmission is thought to occur in a semi-persistent manner (Martelli *et al.*, 2002; Tsai *et al.*, 2008), although Cid *et al.* (2007) have suggested a circulative transmission mechanism. Importantly, first-instar nymphs, unlike other stages that are more sessile, move actively and can be dispersed by wind (Gullan & Kosztarab, 1997; Walker *et al.*, 2004; Grasswitz & James, 2008) and are more efficient vectors than later instars and adult females (Tsai *et al.*, 2008; Sandanayaka *et al.*, 2013).

Both the vine mealybug, *Planococcus ficus* (Signoret), and the longtailed mealybug, *Pseudococcus longispinus* (Targioni Tozzetti) (Hemiptera: Pseudococcidae), are vectors of GLRaV-3 (e.g. Tanne *et al.*, 1989; Engelbrecht

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& Kasdorf, 1990). They occur in many grapevine-growing regions throughout the world, including Africa, Europe and the USA. *Planococcus ficus* is the most abundant species on grapevines in South Africa, whereas *Ps. longispinus* usually only occurs in low numbers and in small patches in vineyards in this country (Walton & Pringle, 2004; Walton *et al.*, 2009).

Several studies have examined aspects of the transmission biology of mealybug vectors of GLRaV-3 (e.g. Cabaleiro & Segura, 1997; Petersen & Charles, 1997; Douglas & Krüger, 2008; Tsai *et al.*, 2008; Mahfoudhi *et al.*, 2009; Tsai *et al.*, 2010; Le Maguet *et al.*, 2012); for reviews see Charles *et al.* (2006), Almeida *et al.* (2013) and Naidu *et al.* (2014). Understanding the transmission biology of GLRaV-3 by mealybug vectors is important for devising management strategies for grapevine leafroll, especially in view of increasing reports of the occurrence of GLRaV-3 and other leafroll-associated viruses (e.g. Akbaş *et al.*, 2007; Cabaleiro *et al.*, 2008; Golino & Almeida, 2008; Fuchs *et al.*, 2009; Almeida *et al.*, 2013). The aim of this study was to determine (i) acquisition and inoculation access periods and (ii) the effects of post-acquisition starving and post-acquisition feeding on the persistence of GLRaV-3 using nymphs of South African populations of *Pl. ficus* and *Ps. longispinus*.

MATERIALS AND METHODS

Insects

Individuals from a laboratory culture of *Pl. ficus*, established with mealybugs collected in vineyards in the Western Cape (South Africa) and maintained for several generations on butternut, a non-host of GLRaV-3, were used. A non-viruliferous culture of *Ps. longispinus* was established on *Alocasia macrorrhizos* L. (Araceae) with specimens collected from greenhouse-grown banana plants in Pretoria (Gauteng, South Africa) and maintained for several generations. Initial attempts to establish a culture on grapevine (cv. Merlot, cv. Cabernet franc) with individuals collected from grapevines in vineyards in the Western Cape failed, as *Ps. longispinus* seemed unable to produce successive generations on this host. The identifications of *Pl. ficus* and *Ps. longispinus* were confirmed by Ian Millar (Biosystematics Division, Agricultural Research Council – Plant Protection Research Institute (ARC-PPRI)) and by using a multiplex polymerase chain reaction (PCR) technique described by Saccaggi *et al.* (2008). Mealybugs were kept in an insect growth room at approximately 25°C, 65% humidity and 14 h:10 h light:dark photoperiod.

Virus source and recipient plants

Plants propagated from stem cuttings of the rootstock hybrid LN33 (1/5/2, ARC Infruitec-Nietvoorbij, South Africa) with GLRaV-3 isolate 621 (Jooste *et al.*, 2010) served as virus source. Plants propagated from stem cuttings of GLRaV-3-free Cabernet franc vines (mixed clones) at ARC Infruitec-Nietvoorbij and Vititec (Pty) Ltd (Western Cape, South Africa) were used as recipient vines. Virus source plants and recipient plants were kept in separate insect-free greenhouse compartments at approximately 25°C, natural humidity and

day-length or environment-controlled growth rooms under the same environmental conditions as the insects.

Acquisition and inoculation access periods

To determine the acquisition access period (AAP) of *Pl. ficus*, first- to second-instar nymphs were initially subjected to acquisition periods ranging from one to seven days on virus source plants (LN33) and then transferred to healthy recipient plants (Cabernet franc) in groups of 50 nymphs per plant for a seven-day inoculation access period (IAP). To determine the IAP, first- to second-instar nymphs were given a seven-day AAP on virus source plants and IAPs of one to seven days on virus-free plants, again in groups of 50 nymphs per plant. Originally, one and seven days were chosen for AAPs and IAPs based on a study by Cabaleiro and Segura (1997), which showed that AAPs of three and seven days, but not one day, were sufficient for *Planococcus citri* (Risso) to transmit GLRaV-3. However, after no apparent difference in transmission efficiency was observed within AAPs or IAPs of one to seven days, nor for the number of nymphs used per group (Douglas & Krüger, 2008), shorter AAPs and IAPs were tested with groups of 15 to 20 nymphs per plant. Virus-free first- to second-instar nymphs of *Pl. ficus* were given AAPs of 15 min to 12 h on the virus source plants and an IAP of four days in groups of 15 to 20 nymphs on the recipient plants. Similarly, groups of 15 to 20 virus-free first- to second-instar nymphs of *Pl. ficus* were given IAPs of 15 min to 12 h on recipient plants after AAPs of four days.

To test the AAPs of *Ps. longispinus*, first- to second-instar nymphs were allowed to feed on virus-infected vines for periods ranging from 30 min to 24 h. Mealybugs were then transferred in groups of 15 to virus-free recipient vines (cv. Cabernet franc) for at least four days. The procedure for testing IAPs was similar to that for testing acquisition access time. Virus-free nymphs were allowed to feed on virus-infected vines for two to five days and then transferred to healthy recipient plants in the manner described above, where they were given inoculation feeding periods ranging from 30 min to 24 h. Nymphs of *Pl. ficus* or *Ps. longispinus* feeding on leaves of plants or on butternut were gently disturbed with a fine paint brush until they stopped feeding. Only these nymphs were carefully transferred to and from plants. For IAPs of 48 h or more, nymphs were transferred from virus source to recipient plants using small leaf cuttings with first- to second-instar nymphs placed on leaves of each of the recipient plants. As the leaf cuttings desiccated, the mealybugs moved to leaves on the plant, usually within the first few hours. Plants exposed to virus-free *Pl. ficus* and *Ps. longispinus* nymphs served as negative controls.

For transmission experiments with *Pl. ficus* and *Ps. longispinus*, five and six plants were used per AAP and IAP respectively. The number of plants used in experiments was limited by the number of pesticide- and virus-free plants available.

To prevent *Pl. ficus* and *Ps. longispinus* nymphs moving between plants and treatments, each plant was placed in its own separate insect-proof cage, and each cage was placed on four saucers containing engine oil to avoid movement of mealybugs between cages. Experiments were undertaken in

insect rearing rooms at approximately 25°C, 65% humidity and 14 h photoperiod. Experiments were replicated using different source plants and by carrying out transmissions on different days, with a negative control plant for each day and each species.

After completion of the transmission experiments, plants were treated with chlorpyrifos and imidacloprid to remove nymphs and to prevent re-infestation. Thereafter, plants were transferred to glasshouses or plant growth rooms, where they were kept at approximately 25°C and natural humidity. In addition to the negative control plants used in the experiments, virus-free grapevine plants were kept in plant growth rooms as additional negative controls throughout the study. Plants were tested for GLRaV-3 starting at the earliest six weeks after treatment, and then at various intervals until two years after treatment. Not all plants used in the transmission experiments survived the dormant period.

GLRaV-3 retention

The transmission of plant viruses by insect vectors is dependent on whether insects have been starving or feeding. The effects of post-acquisition starving and post-acquisition feeding on the retention and subsequent transmission of GLRaV-3 by *Pl. ficus* were determined using first- to second-instar nymphs. Nymphs were given AAPs of four to six days on LN33 cuttings and were then carefully transferred with a fine paint brush in groups of 15 to 20 to one of three treatments: (i) plastic microtubes for starving periods of 6 h to four days (nymphs did not survive for more than four days when starving), (ii) fresh *Ficus benjamini* (Moraceae) leaves kept in glass tubes for feeding on a virus non-host for up to eight days, or (iii) Cabernet franc plants for feeding on virus-susceptible plants for periods of two to eight days. Thereafter, nymphs were transferred to healthy Cabernet franc plants to test their ability to transmit the virus. After completion of the experiments, plants were treated with pesticides as described previously. Subsamples of mealybugs were removed from *F. benjamini* leaves and placed in microtubes and, together with those starved in microtubes, were frozen (-20°C) at the various time intervals for later analysis. Plants exposed to virus-free nymphs served as negative controls. Sample size ranged from three to five plants.

To examine GLRaV-3 retention by *Ps. longispinus*, first- to second-instar nymphs were given AAPs of two to five days on GLRaV-3 source plants. They were then transferred with a fine paint brush in groups of 10 to healthy vines or Eppendorf tubes for post-acquisition feeding or starving periods ranging from 30 min to 72 h. The nymphs were then killed by freezing at -20°C and subsequently tested for GLRaV-3 by nested RT-PCR. Ten virus-free nymphs were collected directly from the *A. macrorrhizos* plant, while 10 nymphs were sampled directly from the virus source plant after an AAP of at least two days to serve as negative and positive controls, respectively. Nymphs of *Pl. ficus* and *Ps. longispinus* were tested individually for the presence of GLRaV-3.

Virus detection

To determine the virus status of the GLRaV-3 source and recipient plants, plant samples were tested for GLRaV-1 to

3, grapevine virus A (GVA) and grapevine virus B (GVB) using nested reverse transcription polymerase chain reaction (nested RT-PCR), an indirect enzyme-linked immunosorbent assay (ELISA) for the simultaneous detection of GLRaV-1, GLRaV-2, and GLRaV-3 in grapevines developed by D. Goszczynski (Virology Unit, ARC-PPRI), and immunosorbent electron microscopy (ISEM) (Milne & Luisoni, 1977). Plants that tested positive for GLRaV-3 only were used as virus source plants. The GLRaV-3 isolate was determined by E. Jooste (Virology Unit, ARC-PPRI).

To ensure that mealybugs were virus free, sub-samples of *Pl. ficus* and *Ps. longispinus* from the laboratory cultures were tested for GLRaV-3 using nested RT-PCR before the transmission experiments. In order to detect GLRaV-3 in individual first- to second-instar nymphs and plant samples, the method described by La Notte *et al.* (1997) was adopted. Nested RT-PCR was performed using the primers developed by Ling *et al.* (2001) and following the protocol adapted from Ling *et al.* (2001) by M. van der Merwe. For details of the extraction and nested RT-PCR, see Douglas and Krüger (2008). Plants were tested from six weeks to two years after GLRaV-3 transmission.

Statistical analysis

The ratios of the total numbers of GLRaV-3-positive plants to the total number of negative plants for the different treatments were analysed using chi-square (χ^2) tests. For the analysis of AAPs and IAPs with *P. ficus* as vector, data on shorter (15 min to 1 h (12 h)) and longer (one to seven days) AAPs and IAPs, respectively, were pooled. The Bonferroni adjustment was used when performing multiple statistical significance tests on the same data (Sokal & Rohlf, 1995). Analyses were carried out with Statistica© (Version 11 Statsoft, Inc. 1984–2012).

RESULTS

Acquisition access and inoculation access times

Planococcus ficus

An AAP or an IAP of 15 min was sufficient to acquire GLRaV-3 or to transmit the virus respectively (Table 1). For AAPs of one to seven days, the transmission rate ranged from 80 to 100%, while transmissions for IAPs of one to seven days ranged from 25 to 80%. Comparing the proportion of infected plants with long AAPs and IAPs (one to seven days) showed that the overall transmission success of IAPs (58%) was significantly lower than that of AAPs (95%) ($\chi^2 = 14.96$, d.f. = 1, $P < 0.001$). For short access periods of 15 min to 1 h, transmission efficiency ranged from 20 to 60% for AAPs and from 20 to 50% for IAPs. The overall transmission success for transmission periods of 1 h or less than 24 h was 38% for AAPs and 31% for IAPs. There was no difference between the total infected plants for AAPs and IAPs for these periods ($\chi^2 = 0.14$, d.f. = 1, $P = 0.7098$). In general, transmission success was higher for AAPs of 24 h or more compared to AAPs of 12 h or less ($\chi^2 = 26.03$, d.f. = 1, $P < 0.001$). IAP transmission success was more variable and did not differ between IAPs of 24 h or more and IAPs of 1 h or less ($\chi^2 = 3.20$, d.f. = 1, $P = 0.0738$).

TABLE 1

Acquisition access periods (AAP) and inoculation access periods (IAP) for transmission of grapevine leafroll-associated virus 3 (GLRaV-3) by *Planococcus ficus* to grapevine plants (cv. Cabernet franc) using groups of 15 to 20 (15 min to 12 h) and 50 (24 h to seven days) first- and second-instar nymphs per plant.

AAP (followed by IAP of four days)	Positive plants/inoculated plants (% infected)	IAP (after AAP of four days)	Positive plants/inoculated plants (% infected)
15 min	1/5 (20)	15 min	2/4 (50)
30 min	3/5 (60)	30 min	1/5 (20)
1 h	2/6 (33)	1 h	2/7 (29)
4 h	1/3 (33)	4 h	-
8 h	1/3 (33)	8 h	-
12 h	2/5 (40)	12 h	-
24 h	6/6 (100)	24 h	3/6 (50)
48 h	5/6 (83)	48 h	1/4 (25)
3 d	5/5 (100)	3 d	4/5 (80)
4 d	5/5 (100)	4 d	4/6 (67)
5 d	6/6 (100)	5 d	4/6 (67)
6 d	4/5 (80)	6 d	2/5 (40)
7 d	6/6 (100)	7 d	4/6 (67)
Negative controls	0/8 (0)	Negative controls	0/8 (0)

TABLE 2

Acquisition access periods (AAP) and inoculation access periods (IAP) for transmission of grapevine leafroll-associated virus 3 (GLRaV-3) by *Pseudococcus longispinus* to grapevine plants (cv. Cabernet franc) using groups of 15 to 20 first- and second-instar nymphs per plant.

AAP (followed by IAP of 4 days)	Positive plants/ inoculated plants (% infected)	IAP (after AAP of 4 days)	Positive plants/ inoculated plants (% infected)
30 min	2/5 (40)	30 min	0/3 (0)
1 h	2/4 (50)	1 h	3/4 (75)
2 h	1/4 (25)	2 h	0/4 (0)
4 h	1/2 (50)	4 h	3/4 (75)
8 h	0/2 (0)	8 h	0/4 (0)
16 h	0/4 (0)	16 h	1/5 (20)
24 h	1/4 (25)	24 h	1/4 (25)
Negative controls	0/5 (0)	Negative controls	0/5 (0)

Pseudococcus longispinus

When feeding on viruliferous vines, *Ps. longispinus* acquired GLRaV-3 within 30 min and was able to transmit the virus to healthy vines (Table 2). The transmission rate for AAPs ranging from 30 min to 24 h ranged from 0 to 50%. An IAP of 1 h, but not one of 30 min, was sufficient to transmit the virus to healthy plants (Table 2). The transmission efficiency for IAPs ranging from 30 min to 24 h ranged from 0 to 75%. There was no difference in the overall transmission success for AAPs (31%) and IAPs (29%) ($\chi^2 = 0.03$, d.f. = 1, $P = 0.8597$).

GLRaV-3 retention

Planococcus ficus

Except for nymphs starving for four and eight days, nymphs under all other experimental conditions were able to transmit GLRaV-3 (Table 3). From 24 to 48 h post-acquisition

feeding, the transmission rate increased from 40 to 100% when feeding on a virus non-host, and from 60 to 100% when feeding on grapevine. However, the transmission rate declined for mealybugs feeding on a virus non-host or grapevine for four and eight days. *Planococcus ficus* could retain the virus for four and eight days when starving and feeding respectively on a non-virus host (Fig. 1). Forty-six percent of nymphs tested positive for GLRaV-3 after an AAP of at least four days. The percentage of GLRaV-3-positive nymphs fluctuated between 14 and 64% when starving, but declined over time from 64 to 9% when feeding on the virus non-host. Only one out of 11 individuals tested positive for GLRaV-3 after eight days on the virus non-host, and that only very weakly. Nevertheless, GLRaV-3 could be transmitted successfully to a healthy grapevine plant by *Pl. ficus* after feeding for eight days on a virus non-host.

TABLE 3

Effect of post-acquisition starving and post-acquisition feeding on the persistence of GLRaV-3 in *Planococcus ficus* using groups of 20 first- to second-instar nymphs per plant and acquisition access periods (AAP) and inoculation access periods (IAP) of four to six days each.

Treatment	Post-acquisition starving/feeding time	Positive plants/inoculated plants (% infected)
Starving	1 d	3/5 (60)
	2 d	3/5 (60)
	4 d	0/3 (0)
	8 d	- -
Non-host	1 d	2/5 (40)
	2 d	4/4 (100)
	4 d	3/4 (75)
	8 d	1/4 (25)
<i>Vitis vinifera</i> (cv. Cabernet franc)	1 d	3/5 (60)
	2 d	3/3 (100)
	4 d	2/4 (50)
	8 d	1/3 (33)
Negative controls		0/4 (0)

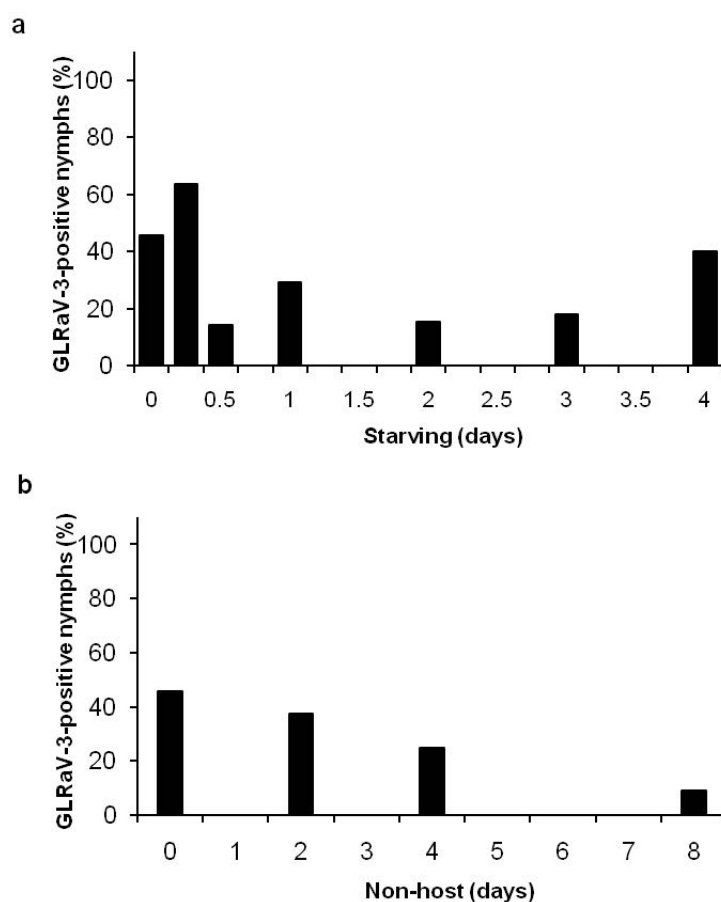


FIGURE 1

Effect of time on GLRaV-3 retention in *Planococcus ficus* nymphs after (a) post-acquisition starving in microtubules (n = 5 - 24) and (b) post-acquisition feeding on *Ficus benjamini*, a virus non-host (n = 8 - 24).

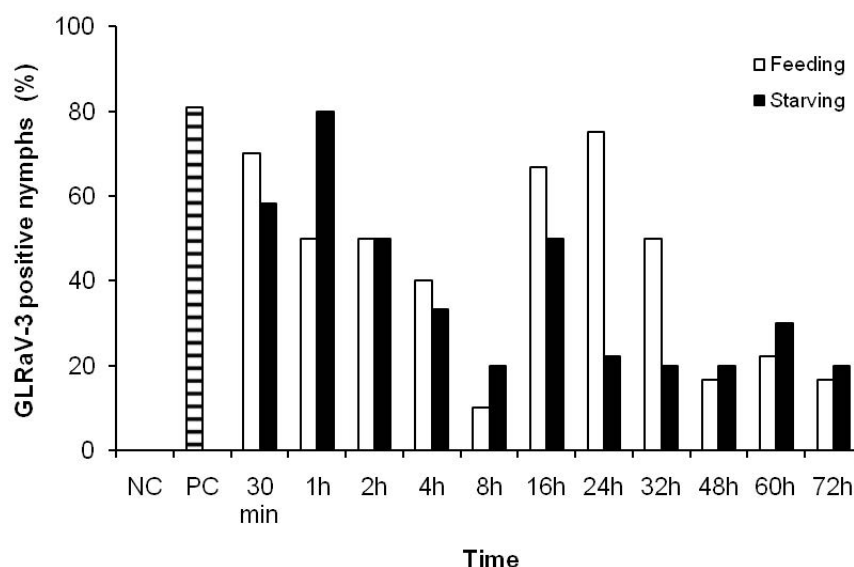


FIGURE 2

Effect of time on GLRaV-3 retention in *Pseudococcus longispinus* nymphs after post-acquisition starving in microtubes and post-acquisition feeding on healthy grapevines (n = 10).

Pseudococcus longispinus

Eighty-one percent of *Ps. longispinus* nymphs tested positive for GLRaV-3 after an AAP of at least two days (Fig. 2). At feeding or starving times ranging from 30 min to 2 h, the virus was retained in at least 50% of the mealybugs. There was no significant difference in retention between feeding and starving mealybugs ($\chi^2 = 6.10$; d.f. = 10; $P > 0.05$). The percentage of infected mealybugs declined over time from 81% to 18% at 72 h, the longest retention time tested. However, the difference between infection rates at different retention times was not significant (feeding: $\chi^2 = 18.30$; d.f. = 10; $P > 0.05$; starving: $\chi^2 = 17.72$; d.f. = 10; $P > 0.05$).

Negative controls

All nymphs collected from butternut (*Pl. ficus*) and *A. macrorrhizos* (*Ps. longispinus*) tested negative for GLRaV-3. For all experiments, plants that served as negative controls, i.e. plants exposed to non-viruliferous nymphs and additional virus-free plants maintained together with the plants used in the experiments, tested negative for GLRaV-3 throughout.

DISCUSSION

It was shown previously that *Pl. ficus* nymphs can acquire or transmit GLRaV-3 after AAPs and IAPs of 1 h (Tsai *et al.*, 2008). The results of this study show that *Pl. ficus* nymphs can acquire GLRaV-3 in 15 min and transmit the virus to healthy plants, and that viruliferous nymphs can inoculate healthy plants within 15 min. This is in accordance with other Hemiptera-transmitted Closteroviridae (Martelli & Candresse, 2014). Previous work on the transmission of GLRaV-3 by *Ps. longispinus* was done with AAPs or IAPs of one day or more (Petersen & Charles, 1997; Golino *et al.*, 2002; Kuniyuki *et al.*, 2005; Douglas & Krüger, 2008). In

the current study, *Ps. longispinus* was able to transmit the virus to a healthy plant after an AAP of 30 min and an IAP of 1 h or more. The short AAPs and IAPs for *Pl. ficus* and *Ps. longispinus* nymphs are in contrast to the 24 h minimum feeding time observed for adult *Ps. longispinus* in a study on stylet penetration behaviour (Sandanayaka *et al.*, 2013), and the average of 6 h required by *Pl. citri* to reach the phloem sap (Cid & Fereres, 2010).

The results of this study further show that the transmission success of GLRaV-3 by *Pl. ficus* nymphs is lower with AAPs of 12 h or less compared with AAPs of at least 24 h, when transmission success reached 100%. Similarly, Tsai *et al.* (2008) observed that transmission success peaked at 24 hours. The results for *Ps. longispinus* and IAPs of *Pl. ficus* in the current study were more variable and no clear trends were discernible. However, nymphs lose the ability to transmit GLRaV-3 after moulting. It is possible that first-instar nymphs moulted during the long AAPs of four to six days, which may have affected transmission success.

After an acquisition period of at least two days, 81% of single nymphs of *Ps. longispinus* exposed to viruliferous grapevines tested positive for the presence of GLRaV-3. This is a high infection rate when compared to the 46% of *Pl. ficus* observed in this study, and infection rates of 23% for *Pl. ficus* (Mahfoudhi *et al.*, 2009) and 13 to 19% for *Pl. citri* (Cabaleiro & Segura, 1997). However, a previous study has shown that single first-instar nymphs of *Pl. ficus* and *Ps. longispinus* can transmit GLRaV-3 with equal efficiency (Douglas & Krüger, 2008).

GLRaV-3 has been categorised as a semi-persistently transmitted virus (Cabaleiro & Segura, 1997; Tsai *et al.*, 2008). The results of the current study show that acquisition and inoculation periods lie in the range of 15 min to 1 h. There is no latent period, the virus is lost during moult-

ing and is not transmitted transovarially (Tsai *et al.*, 2008). These findings agree with the classification of GLRaV-3 as a semi-persistently transmitted virus. However, the current study has shown that, after feeding on a non-host for eight days, *Pl. ficus* was able to transmit GLRaV-3 to healthy vines. This is longer than the retention periods of three days previously reported for this species (Tsai *et al.*, 2008), or the 24 h for *Pl. citri* (Cabaleiro & Segura, 1997), a species closely related to *Pl. ficus* (Downie & Gullan, 2004), when feeding on a non-host. It is possible that the nymphs did not moult – no exuviae were observed – due to slow growth on a host less suitable than grapevine. Generally, however, the reason for the long retention times is not understood (Martelli & Candresse, 2014).

The shorter AAPs and IAPs, higher transmission efficiencies and longer retention time observed in this study in comparison to Cabaleiro and Segura (1997) and Tsai *et al.* (2008) could be a reflection of experimental conditions, e.g. virus titre in source plants, sensitivity of detection method or GLRaV-3 isolate used. Further experiments are needed to explain the long retention time of eight days and to determine whether the GLRaV-3 isolate 621, one of the most common of the isolates recorded on grapevine in South Africa (Jooste *et al.*, 2010), is more efficiently transmitted by *Pl. ficus* than less common isolates.

CONCLUSIONS

Planococcus ficus nymphs can acquire GLRaV-3 after an AAP of as little as 15 min and transmit the virus to healthy plants, and they can inoculate healthy plants after an IAP of 15 min. The nymphs retained the virus for at least eight days when feeding on a non-virus host or on virus-free grapevines, and for at least two days when starving, after which they still were able to transmit GLRaV-3 to virus-free grapevines. *Pseudococcus longispinus* nymphs can transmit the virus to healthy plants after an AAP of 30 min and an IAP of 1 h or more. They can retain GLRaV-3 for at least three days when starving or feeding on virus-free grapevines. The short virus acquisition times, together with the long retention times, merit further investigation. First- and second-instar nymphs are the most mobile and easily dispersible life stages of mealybugs, and a single nymph can successfully transmit GLRaV-3 to a healthy grapevine plant. In view of these findings, this study has serious implications for grapevine leafroll management strategies that rely on effective vector control.

LITERATURE CITED

- Akbaş, B., Kunter, B. & İlhan, D., 2007. Occurrence and distribution of grapevine leafroll-associated viruses 1, 2, 3 and 7 in Turkey. *J. Phytopathol.* 155, 122-124.
- Almeida, R.P.P., Daane, K.M., Bell, V.A., Blaisdell, G.K., Cooper, M.L., Herrbach, E. & Pietersen, G., 2013. Ecology and management of grapevine leafroll disease. *Front. Microbiol.* 4 (Article 94), 1-13.
- Atallah, S.S., Gómez, M.I., Fuchs, M.F. & Martinson, T.E., 2012. Economic impact of grapevine leafroll disease on *Vitis vinifera* cv. Cabernet franc in Finger Lakes vineyards of New York. *Am. J. Enol. Vitic.* 63, 73-79.
- Belli, G., Fortusini, A., Casati, P., Belli, L., Bianco, P.A. & Prati, S., 1994. Transmission of a grapevine leafroll associated closterovirus by the scale insect *Pulvinaria vitis* L. *Riv. Pat. Veg. S. V.* 4, 105-108.
- Cabaleiro, C. & Segura, A., 1997. Some characteristics of the transmission of grapevine leafroll associated virus 3 by *Planococcus citri* Risso. *Eur. J. Plant Pathol.* 103, 373-378.
- Cabaleiro, C., Couceiro, C., Pereira, S., Cid, M., Barrasa, M. & Segura, A., 2008. Spatial analysis of epidemics of grapevine leafroll associated virus-3. *Eur. J. Plant Pathol.* 121, 121-130.
- Cabaleiro, C., Segura, A. & García-Berrios, J.J., 1999. Effects of grapevine leafroll-associated virus 3 on the physiology and must of *Vitis vinifera* L. cv. Albariño following contamination in the field. *Am. J. Enol. Vitic.* 50, 40-44.
- Charles, J.G., Cohen, D., Walker, J.T.S., Forgie, S.A., Bell, V.A. & Breen, K.C., 2006. A review of the ecology of grapevine leafroll associated virus type 3 (GLRaV-3). *N. Z. Plant Protect.* 59, 330-337.
- Cid, M. & A. Fereres, A., 2010. Characterization of the probing and feeding behavior of *Planococcus citri* (Hemiptera: Pseudococcidae) on grapevine. *Ann. Entomol. Soc. Am.* 103, 404-417.
- Cid, M., Pereira, S., Cabaleiro, C., Faoro, F. & Segura, A., 2007. Presence of grapevine leafroll-associated virus 3 in primary salivary glands of the mealybug vector *Planococcus citri* suggests a circulative transmission mechanism. *Eur. J. Plant Pathol.* 118, 23-30.
- Douglas, N. & Krüger, K., 2008. Transmission efficiency of grapevine leafroll-associated virus 3 (GLRaV-3) by the mealybugs *Planococcus ficus* and *Pseudococcus longispinus* (Hemiptera: Pseudococcidae). *Eur. J. Plant Pathol.* 122, 207-212.
- Downie, D.A. & Gullan, P.J., 2004. Phylogenetic analysis of mealybugs (Hemiptera: Coccoidea: Pseudococcidae) based on DNA sequences from three nuclear genes, and a review of the higher classification. *Syst. Entomol.* 29, 238-259.
- Engelbrecht, D.J. & Kasdorf, G.G.F., 1990. Transmission of grapevine leafroll disease and associated closteroviruses by the vine mealybug, *Planococcus ficus*. *Phytophylactica* 22, 341-346.
- Fuchs, M., Martinson, T.E., Loeb, G.M. & Hoch, H.C., 2009. Survey for the three major leafroll disease-associated viruses in Finger Lakes vineyards in New York. *Plant Dis.* 93, 395-401.
- Goheen, A.C. & Cook, J.A., 1959. Leafroll (red-leaf or rogeau) and its effects on vine growth, fruit quality, and yields. *Am. J. Enol. Vitic.* 10, 173-181.
- Golino, D.A. & Almeida, R., 2008. Studies needed of vectors spreading leafroll disease in California vineyards. *Calif. Agric.* 62, 174.
- Golino, D.A., Sim, S.T., Gill, R. & Rowhani, A., 2002. California mealybugs can spread grapevine leafroll disease. *Calif. Agric.* 56, 196-201.
- Grasswitz, T.R. & James, D.G., 2008. Movement of grape mealybug, *Pseudococcus maritimus*, on and between host plants. *Entomol. Exp. Appl.* 129, 268-275.
- Gullan, P.J. & Kosztarab, M., 1997. Adaptations in scale insects. *Annu. Rev. Entomol.* 42, 23-50.
- Jooste, A.E.C., Maree, H.J., Bellstedt, D.U., Goszczynski, D.E., Pietersen, G. & Burger, J.T., 2010. Three genetic grapevine leafroll-associated virus 3 variants identified from South African vineyards show high variability in their 5'UTR. *Arch. Virol.* 155, 1997-2006.
- Krüger, K. & Douglas-Smit, N., 2013. Grapevine leafroll-associated virus 3 (GLRaV-3) transmission by three soft scale insect species (Hemiptera: Coccidae) with notes on their biology. *Afr. Entomol.* 21, 1-8.

- Kuniyuki, H., Rezende, J.A.M., De Willink, C.G., Novo, J.P.S. & Yuki, V.A., 2005. Transmissão do grapevine leafroll-associated virus 3 pela cochonilha *Pseudococcus longispinus* Targioni-Tozzetti (Hemiptera: Pseudococcidae). *Summa Phytopathol.* 31, 65-68.
- La Notte, P., Minafra, A. & Saldarelli, P., 1997. A spot-PCR technique for the detection of phloem-limited grapevine viruses. *J. Virol. Methods* 66, 103-108.
- Le Maguet, J., Beuve, M., Herrbach, E. & Lemaire, O., 2012. Transmission of six ampeloviruses and two vitiviruses to grapevine by *Phenacoccus aceris*. *Phytopathol.* 102, 717-723.
- Ling, K.-S., Zhu, H.-Y., Petrovic, N. & Gonsalves, D., 2001. Comparative effectiveness of ELISA and RT-PCR for detecting grapevine leafroll-associated closterovirus-3 in field samples. *Am. J. Enol. Vitic.* 52, 21-27.
- Mahfoudhi, N., Digiario, M. & Dhoubi, M.H., 2009. Transmission of grapevine leafroll viruses by *Planococcus ficus* (Hemiptera: Pseudococcidae) and *Ceroplastes rusci* (Hemiptera: Coccidae). *Plant Dis.* 93, 999-1002.
- Martelli, G.P., 1986. Virus and virus-like diseases of the grapevine in the Mediterranean area. *FAO Plant Prot. Bull.* 34, 25-42.
- Martelli, G.P. & Candresse, T., (2014) Closteroviridae. In: *Encyclopedia of Life Sciences (eLS)*. John Wiley & Sons Ltd, Chichester. <http://www.els.net> [doi: 10.1002/9780470015902.a0000747.pub3]
- Martelli, G.P., Agranovsky, A.A., Bar-Joseph, M., Boscia, D., Candresse, T., Coutts, R.H.A., Dolja, V.V., Falk, B.W., Gonsalves, D., Jelkmann, W., Karasev, A.V., Minafra, A., Namba, S., Vetten, H.J., Wisler, G.C. & Yoshikawa, N., 2002. The family *Closteroviridae* revised. *Arch. Virol.* 147, 2039-2044.
- Martin, R.R., Eastwell, K.C., Wagner, A., Lamprecht, S. & Tzanetakis, I.E., 2005. Survey for viruses of grapevine in Oregon and Washington. *Plant Dis.* 89, 763-766.
- Milne, R.G. & Luisoni, E., 1977. Rapid immune electron microscopy of virus preparation. In: Maramorosch, K. & Koprowski, H., eds: *Methods in Virology*, vol 6. Academic Press, New York. pp. 265 – 281.
- Naidu, R., Rowhani, A., Fuchs, M., Golino, D. & Martelli, G.P., 2014. Grapevine leafroll: A complex viral disease affecting a high-value fruit crop. *Plant Dis.* 98, 1172-1185.
- Petersen, C.L. & Charles, J.G., 1997. Transmission of grapevine leafroll-associated closteroviruses by *Pseudococcus longispinus* and *P. calceolariae*. *Plant Pathol.* 46, 509-515.
- Pietersen, G., 2006. Spatio-temporal distribution dynamics of grapevine leafroll disease in Western Cape vineyards. In: *Extended abstracts of the 15th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine*, April 2006, Stellenbosch, South Africa. pp. 126 – 127.
- Pietersen, G., Spreeth, N., Oosthuizen, T., Van Rensburg, A., Van Rensburg, M., Lottering, D., Rossouw, N. & Tooth, D., 2013. Control of grapevine leafroll disease spread at a commercial wine estate in South Africa: A case study. *Am. J. Enol. Vitic.* 64, 296-305.
- Saccaggi, D.L., Krüger, K. & Pietersen, G., 2008. A multiplex PCR assay for the simultaneous identification of three mealybug species (Hemiptera: Pseudococcidae). *Bull. Entomol. Res.* 98, 27-33.
- Sandanayaka, W.R.M., Blouin, A.G., Prado, E. & Cohen, D., 2013. Stylet penetration behaviour of *Pseudococcus longispinus* in relation to acquisition of grapevine leafroll virus 3. *Arthropod-Plant Interact.* 7, 137-146.
- Sokal, R.R. & Rohlf, F.J., 1995 (3rd ed). *Biometry*. W.H. Freeman and Company, New York.
- Tanne, E., Ben-Dov, Y. & Raccach, B., 1989. Transmission of closterolike particles associated with grapevine leafroll by mealybugs (Pseudococcidae) in Israel. In: *Proc. 9th meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine (ICVG)*, September 1987, Kiryat Anavin, Israel. pp. 71 – 73.
- Tsai, C.-W., Chau, J., Fernandez, L., Bosco, D., Daane, K.M. & Almeida, R.P.P., 2008. Transmission of grapevine leafroll-associated virus 3 by the vine mealybug (*Planococcus ficus*). *Phytopathol.* 98, 1093-1098.
- Tsai, C.-W., Rowhani, A., Golino, D.A., Daane, K.M. & Almeida, R.P.P., 2010. Mealybug transmission of grapevine leafroll viruses: An analysis of virus-vector specificity. *Phytopathology* 100, 830-834.
- Walker, J.T.S., Charles, J.G., Froud, K.J. & Connolly, P., 2004. Leafroll virus in vineyards: Modelling the spread and economic impact. *Hort Research Client Report No.* 12795, 1-19.
- Walton, V.M. & Pringle, K.L., 2004. A survey of mealybugs and associated natural enemies in vineyards in the Western Cape Province, South Africa. *S. Afr. J. Enol. Vitic.* 25, 23-25.
- Walton, V.M., Krüger, K., Saccaggi, D.L. & Millar, I.M., 2009. A survey of scale insects (Sternorrhyncha: Coccoidea) occurring on table grapes in South Africa. *J. Insect Sci.* 9 (Article 47), 1-6.